

# A novel MLSA allelic profile 'A15' of *Mycoplasma mycoides* subsp *mycoides* in Niger

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## ABSTRACT

*Mycoplasma mycoides* subsp. *mycoides* (*Mmm*) is the aetiological agent of contagious bovine pleuropneumonia (CBPP). The aim of the present study was to identify the profiles of the *Mmm* strains isolated in Niger using the 'Multilocus Sequence Analysis' (MLSA) typing technique based on polymorphism analysis of housekeeping and non-coding genes. The investigation was conducted on samples (n=22) comprising of lung tissues, lymph node and pleural fluids. Following classical PCR, *Mmm* positive amplicons (n=6) were identified. These positive amplicons were then amplified using eight loci of the PG1 reference strain (Loc-PG1-0001, Loc-PG1-0103, Loc-PG1-0287, Loc-PG1-0431, Loc-PG1-0489, Loc-PG1-0523, Loc-PG1-0710 and Loc-PG1-0827). Sequencing followed by the determination of the profile of each strain by the combination of the allele numbers revealed three different MLSA profiles namely; A11, E01 and A15. The profiles A11 and E01 were previously identified. The novel profile identified in this study was named profile A15. The difference was detected while comparing sequences of non-coding loci. This novel profile was named 'A15' according to the similarities with African reference strain profile 'A00' at the seven loci level (loc-0103, loc-0287, loc-0431, loc-0489, loc-0523, loc-0710 and loc-0827). For CBPP control measures, identification and molecular characterization of *Mmm* strains is very important. Thus, the use of MLSA technique is relevant to identify profiles of *Mmm* circulating in Niger. Other countries where CBPP is still endemic are encouraged to use a MLSA scheme to address this issue and, most importantly, to rapidly trace back the origin of outbreaks, which will help reduce the transmission and spread of the disease. In addition, mapping the profiles of strains circulating in each of the countries of the sub-region is necessary for effective control of CBPP.

## KEYWORDS

CBPP, characterization, MLSA /Niger, *Mmm*, PCR

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## 1 | INTRODUCTION

Like many west African countries, outbreaks of contagious bovine pleuropneumonia (CBPP; an economically important disease of cattle) are often reported in Niger.

The causative agent of CBPP is *Mycoplasma mycoides* subsp. *mycoides*, previously named *Mycoplasma mycoides* subsp. *mycoides* Small Colony (SC) type (*Mmm*SC; Manso-Silván et al., 2009). This bacterium belongs to the class of Mollicutes. It is characterized by a small size of their genomes (between 580 and 2000 kb). A large number of mollicutes infect animals, with initial colonization of mucosal surfaces such as the respiratory and urogenital tracts. In livestock, diseases caused by mollicutes have a great economic impact especially for cattle, small ruminants and industrial poultry (Barré et al., 2004).

Transmission of the disease requires close contact between infected and susceptible animals (Niáng et al., 2004; YaYa et al., 1999). CBPP is one of the major diseases of cattle that causes significant losses to both the local and national economy. The serological survey of CBPP in the region of Niamey out of the eight regions of Niger country using c-ELISA revealed an individual seroprevalence of 6.8% (Yansambou, Ferreira, et al., 2018). Furthermore, CBPP endemic situation was confirmed by a seroprevalence study conducted in all regions of the country, revealed an individual prevalence of 4.15% (Yansambou, Diallo, et al., 2018). To reduce the impact of this disease, the government of Niger has implemented an annual policy of free vaccination. Despite this, outbreaks are still reported. In cognisance of this, the propose of this study was to provide the information on the circulation of *Mmm* profiles in Niger through the genetic characterisation of the bacterium from field samples collected between 1990 and 2017. In order to achieve this purpose, molecular typing technique was used in this study.

Multilocus sequence typing (MLST) is a molecular biology technique used for typing multiple loci by allowing the analysis of polymorphisms in housekeeping genes. It was described on *Neisseria meningitidis*, as a microbial typing method (Maiden et al., 1998). However, *Mmm* has a very low variability at the genome level. Therefore, MLST was unsuitable for typing such micro-organisms. Consequently, multilocus sequence analysis (MLSA) technique was developed to establish the differences and similarities of the strains according to non-coding sequences analysis (Lorenzon et al., 2003). This method constitutes a robust tool for the characterization of bacterial species phylogenies (Yaya et al., 2008). The MLSA technique performed in 2003, was reinforced in 2008, by identifying 31 *Mmm* profiles against 15 (Lorenzon et al., 2003; Yaya et al., 2008). Therefore, the MLSA system can be useful for countries at risk of infection or resurgence of CBPP. It can also be suitable for countries (developing countries) committed in the eradication of this disease to trace back the origin of the infection.

## 2 | MATERIAL AND METHODS

### 2.1 | Study area

Niger is a vast Sahelian country covering an area of 1,267,000 km<sup>2</sup>, located in West Africa. It is bordered by Algeria and Libya to the North, by Nigeria and Benin to the South, by Chad to the East and by Mali and Burkina Faso to the West. The country is divided into eight regions (i.e., Agadez, Diffa, Dosso, Maradi, Niamey, Tahoua, Tillabéri and Zinder; Figure 1). Livestock farming is more practiced in the south part of the country. It is mainly comprised of cattle, goats and sheep; but some farmers possess also camels; donkeys and horses. (Merrey & Sally, 2004). In Niger, the cattle population is estimated to be >10 million (MAG/EL., 2017), which immensely contribute to the livelihood of farmers.

### 2.2 | Sample collection

Clinical samples ( $n = 22$ ) comprising of lung tissues ( $n = 15$ ), lymph node ( $n = 1$ ) and pleural fluids ( $n = 6$ ), suspected of being infected by *Mmm* were collected from the field by the veterinary services and transported on ice to the Laboratoire Central de l'Élevage (LABOCEL) Niamey for confirmatory diagnosis. These samples were collected from four of the eight regions of Niger (i.e. Diffa, Dosso, Tahoua and Tillabéri) between 1990 and 2017.

### 2.3 | Sample processing and DNA extraction

Homogenates were prepared by grinding lung and lymph node tissues in DEPC (Diethyl pyrocarbonate) treated, sterile filtered water, placed in individual sterile petri-dishes. The suspensions from the

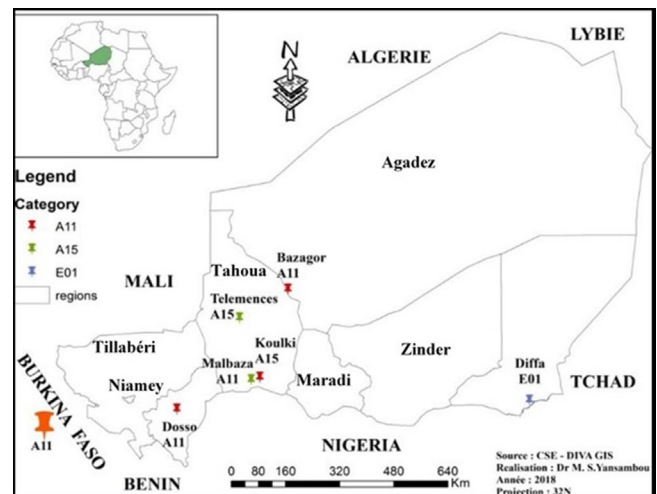


FIGURE 1 Map of Niger showing the distribution of *Mycoplasma mycoides* subsp. *mycoides*

lung, lymph node tissues and the pleural fluids were run at 1,000 g for 5 min and the supernatants were collected in sterile tubes. Two hundred microliters of each sample were used for DNA extraction using the Qiagen DNA extraction kit following the manufacturer's instructions.

## 2.4 | Detection of *Mmm* by polymerase chain reaction

Polymerase chain reaction (PCR) was performed in a 50- $\mu$ l reaction mixture containing: 1X PCR buffer 10X with 25 mM MgCl<sub>2</sub>, 1.25 unit of Qiagen-Taq DNA polymerase (5 U/ $\mu$ l), 300  $\mu$ M of dTTP and dATP, 150  $\mu$ M of dGTP and dCTP, 0.4  $\mu$ M of each MSC1/MS2 primers developed in 1996 by Dedieu et al. (1996) and 2  $\mu$ l of DNA sample. Amplifications were performed using 'Applied Biosystems' (AB) thermal cycler. The thermal cycling was performed according to the initial denaturation step at 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 52°C for 30 s and extension at 72°C for 1 min. The final extension step was maintained at 72°C for 5 min. The size and purity of the amplicons were checked by electrophoretic separation in 2% agarose gels.

## 2.5 | Genotyping *Mmm* by multi locus sequence analysis

The Multilocus Sequence Analysis (MLSA) was used to genotype *Mmm* according to the protocol developed in 2008 by Yaya et al. (2008).

## 2.6 | Amplification of different loci

PCR for the different loci amplification was carried out in a 50  $\mu$ l reaction containing: 1X PCR buffer, 1.25 unit of Qiagen-Taq DNA polymerase (5 U/ $\mu$ l), 1.5 mM MgCl<sub>2</sub>, 300  $\mu$ M of dTTP and dATP, 150  $\mu$ M of dGTP and dCTP, 0.4  $\mu$ M of each primer and 2  $\mu$ l of each DNA sample. Amplifications were performed using 'Applied Biosystems' (AB) thermal cycler. The thermal cycling was performed according to the initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 15 s, annealing at 52°C for 30 s and extension at 72°C for 90 s. The final extension step was maintained at 72°C for 7 min.

The size and purity of the amplicons were checked by electrophoretic separation in 2% agarose gels. The primers used for the amplification of the different loci are summarized in Table 1.

## 2.7 | Allelic profile analysis

Amplicons with relevant features were purified for sequencing using Wizard<sub>SV</sub> Gel and PCR Clean-Up System (Promega-Madison) following the manufacturer's instructions and sent for sequencing at 'LGC Genomics GmbH' (Berlin, Germany) using standard Sanger methods. Each amplicon was sequenced in both forward and reverse strands. The sequencing results obtained with the different primer pairs were assembled using the Staden 2.0.0b8 Package (Pregap4 and Gap4). The sequences obtained from the different amplicons for each locus were aligned and analysed using BioEdit 7.2 software.

The polymorphic sites were checked for possible differences with the reference sequences. A new allele number has been assigned

**TABLE 1** List of primers used for PCR and sequencing based on reference strain PG1 sequence

Loci names	Primers	Sequences (5'-3')	Amplicon size (bp)
Loc-PG1-0001	Loc-PG1-0001-F	AACAAAAGAGATCTTAAATCACACTTTA	538
	Loc-PG1-0001-R	CCTCTTGTTTAACTTCTAGATCAGAAT	
Loc-PG1-0103	Loc-PG1-0103-F	GATGGATATAATCTATACTAGCATTTA	1,321
	Loc-PG1-0103-R	CCTTATATAGATAAACTCCTCCTTA	
Loc-PG1-0287	Loc-PG1-0287-F	GATTGCTTTAATCAATTTCTTACTGA	545
	Loc-PG1-0287-R	GGATAACCTTGATTTTTTATTGCTTTA	
Loc-PG1-0431	Loc-PG1-0431-F	CAATTCTTTAAATTTGGGTTTGTT	608
	Loc-PG1-0431-R	CTTGCAAGAGTATTTAGATTTGATTA	
Loc-PG1-0489	Loc-PG1-0489-F	GTTAGTTGTTGAAATGTTAGATAT	756
	Loc-PG1-0489-R	CCCATATCAGTTTGGATTAA	
Loc-PG1-0523	Loc-PG1-0523-F	ACAGCATTTGATCAAGATTTAAGTAGTT	824
	Loc-PG1-0523-R	TTACCTAGGTGTTTAAACTTCATTTG	
Loc-PG1-0710	Loc-PG1-0711-F	CCAGTTGAACCATTTATTTTATATACCT	643
	Loc-PG1-0711-R	AAATATAAGTGGTCTGGAATAACA	
Loc-PG1-0827	Loc-PG1-0827-F	AGTTGTACAACCTGATGAATCTATGATTAT	619
	Loc-PG1-0827-R	CAGGATATACTTCAAAAATTAAGGTTT	

Source: (Yaya et al., 2008).

**TABLE 2** List of CBPP suspected samples and various allelic profiles obtained using MLSA analyses of the eight loci

Samples	Locality	Year of sampling	Nature of the sample	PCR result	Eight loci profiles from MLSA analysis								Profiles	
					Loc-0001	Loc-0103	Loc-0287	Loc-0431	Loc-0489	Loc-0523	Loc-0710	Loc-0827		
1	Tahoua (Malbaza)	2009	Pleural fluid	+	2	2	4	1	2	2	1	1	1	A11
2	Dosso (Dioundou)	1997	Lung	-										
3	Tahoua (Koulki)	1990	Lung	+	7 <sup>a</sup>	2	1	1	2	1	1	1	1	A15 <sup>a</sup>
4	Dosso (Dioundou)	2002	Lung	-										
5	Dosso (Malgorou)	2002	Lung	-										
6	Diffa	1993	Lung	-										
7	Diffa	1990	Pleural fluid	+	3	2	1	1	1	1	1	1	1	E01
8	Diffa	1993	Pleural fluid	-										
9	Diffa (Bosso)	1993	Lung	-										
10	Tahoua (Bazagor)	2012	Pleural fluid	+	2	2	4	1	2	2	1	1	1	A11
11	Tahoua (Télemcès)	2000	Pleural fluid	+	7 <sup>a</sup>	2	1	1	2	1	1	1	1	A15 <sup>a</sup>
12	Tahoua (Bazagor)	2012	Pleural fluid	-										
13	Tahoua	2009	Lung	-										
14	Dosso	2016	Lung	+	2	2	4	1	2	2	1	1	1	A11
15	Dosso (Bingou)1	2002	Lung	-										
16	Dosso (Bingou)2	2002	Lung	-										
17	Tahoua (Koulki)	1990	Lung	-										
18	Tahoua (Abalak)	2006	Lung	-										
19	Tahoua (Abalak)	2006	Lymph node	-										
20	Tillabéri (Bankilaré)	2017	Lung	-										
21	Tillabéri (Tammou)	2017	Lung	-										
22	Tillabéri	2016	Lung	-										

Note +: Positive, - Negative.

<sup>a</sup>New finding.

each time there is a nucleotide change in the sequence. The profile of each amplicon was determined by combining the allele numbers of the eight loci of the PG1 reference strain (Loc-PG1-0001, Loc-PG1-0103, Loc-PG1-0287, Loc-PG1-0431, Loc-PG1-0489, Loc-PG1-0523, Loc-PG1-0710 and Loc-PG1-0827). The nucleotide reference sequences of the eight loci already listed are obtained with previous studies (Yaya et al., 2008). Due to the lack of online MLSA database like for PubMLST, the results obtained were sent to CIRAD (Centre de Coopération Internationale de la Recherche Agronomique pour le Développement, Montpellier/France), the OIE reference laboratory of CBPP for confirmation and registration of the novel profile identified in this study.

### 3 | RESULTS

#### 3.1 | Detection of *Mmm* by PCR

Of the 22 samples analysed, 6 (6/22) positive amplicons were obtained (Table 2). The electrophoresis on 2% agarose gels showed the six positive amplicons with the expected size (275 bp; Figure 2). These positive amplicons (1–6) correspond respectively to the samples 1, 3, 7, 10, 11 and 14 (Table 2).

#### 3.2 | Amplification of eight loci

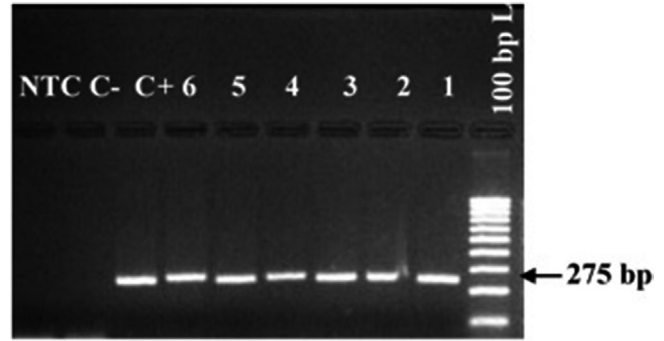
The six positive amplicons were further analysed by amplification of the eight interested loci with their corresponding primers (Table 1). The electrophoresis on 2% agarose gel showed the eight loci with expected sizes of sample 1 (loc-0001: 538 bp, loc-0103: 1,321 bp, loc-0287: 545 bp, loc-0431: 608 bp, loc-0489: 756 bp, loc-0523: 824 bp, loc-0711: 643 bp and loc-0827: 619 bp; Figure 3).

#### 3.3 | Allelic profile analysis

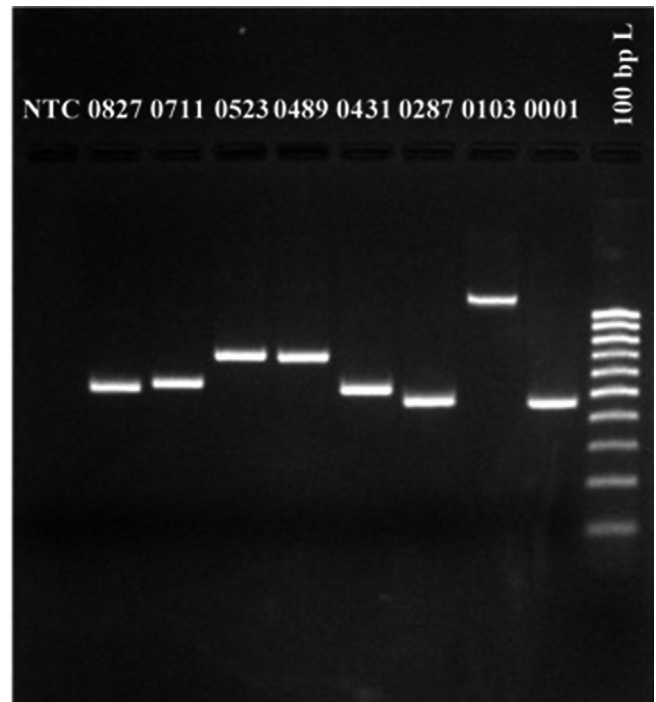
The results revealed the circulation of profile A11, profile E01 and a novel profile. With the collaboration of CIRAD, the novel profile was named profile A15 according to the new allele obtained while combining alleles numbers of the eight loci (Table 2). Profile A11 was located in the regions of Dosso and Tahoua, profile E01 in the region of Diffa and profile A15 in the region of Tahoua (Figure 1).

Allelic sequence analysis of positive amplicons corresponding to samples 3 and 11 are closely related to the allele 4 of loc-0001 (loc-PG1-0001-A14; Figure 4). The difference is due to a nucleotide mutation by substitution. The 'G' (Guanine) was replaced by 'A' (Adenine; c. 282 G > A; Figure 5).

This novel profile A15 showed similarities also with African reference strain profile 'A00' at the seven loci level (i.e. loc-0103, loc-0287, loc-0431, loc-0489, loc-0523, loc-0710 and loc-0827; Table 3).



**FIGURE 2** Gel image showing the six *Mycoplasma mycoides* subsp *mycoides* (*Mmm*) positive amplicons. 100 base pairs Ladder, 1–6: *Mmm* positive amplicons, C+: Positive control, C–: Negative control, NTC: No Template Control)



**FIGURE 3** Gel image showing the amplification of given *Mycoplasma mycoides* subsp *mycoides* (*Mmm*) DNA sample in all loci. 100 bp L: 100 base pairs Ladder, loc-0001:538 bp, loc-0103:1,321 bp, loc-0287:545 bp, loc-0431:608 bp, loc-0489:756 bp, loc-0523:824 bp, loc 0,711:643 bp and loc-0827:619 bp. NTC: No Template Control

### 4 | DISCUSSION

This study reports the first MLSA analysis of *Mmm* in Niger. Among the samples ( $n = 22$ ) analysed, only six (6/22) were confirmed positive by PCR. It is possible that the poor quality of some of the samples collected led to false negative PCR results or these animals manifesting symptoms of CBPP were actually suffering from other like CBPP respiratory diseases.

Most of the positive amplicons (5/6) were from the west part of the country (i.e., Tahoua and Dosso). Only one was from the extreme

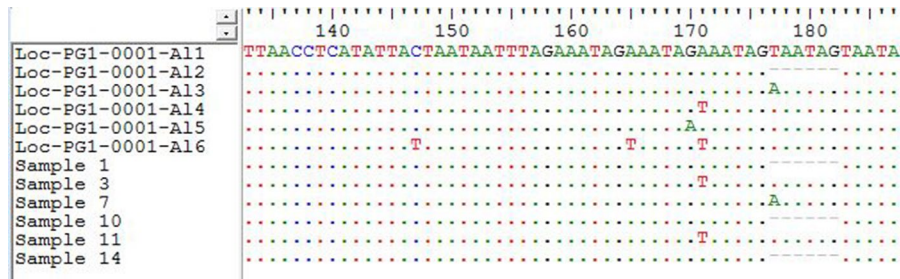


FIGURE 4 Sequences of the loc-0001 locus from nucleotide 133 to 187 of the Niger's samples with the six reference alleles

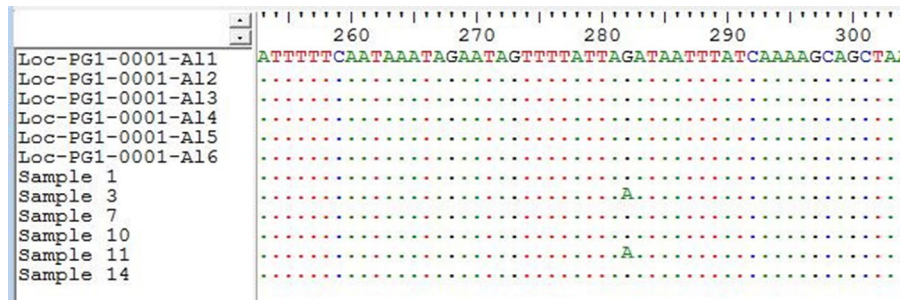


FIGURE 5 Sequences of the loc-0001 locus from nucleotide 253 to 303 of the Niger's samples with the six reference alleles

Profiles	Loc-0001	Loc-0103	Loc-0287	Loc-0431	Loc-0489	Loc-0523	Loc-0710	Loc-0827
A15 <sup>b</sup>	7 <sup>a</sup>	2	1	1	2	1	1	1
A00	1	2	1	1	2	1	1	1

TABLE 3 Similarities between A15 and A00 MLSA allelic profiles of *Mmm*

<sup>a</sup>New allele found.

<sup>b</sup>New allelic profile found.

south-east part (Diffa). Due to the limited samples analysed, this study provides an idea on how *Mmm* profiles were distributed within the eight regions of the country.

According to the Yaya's study in 2008, for the eight loci selected for MLSA, 31 allelic profiles have been identified. These profiles are divided into seven groups: A, B, C, D, E, F and G. The largest group in number of isolates was group A with 13 different profiles (A00, A01, A02, A03, A04, A05, A06, A07, A08, A09, A10, A11 and A12). Profiles 'A' are African ones, especially from west Africa. In this present study, *Mmm* strains circulating in Niger belongs to 3 MLSA allelic profiles. These profiles are A11, E01 and A15. Half of the *Mmm* positive amplicons (3/6) belongs to profile A11. The profile A11 has been identified first in Burkina Faso in 2005 (Yaya, 2008). It was also identified in recent outbreaks in Senegal (data not show) and in Niger in this present study. This profile seems to be widespread and could be explained by transboundary movement between Sahelian countries. This risk factor is important in the spread of CBPP and explain the difficulties of the disease control in these neighbouring countries (Touré et al., 2012).

The previous study (Yaya et al., 2008) also identified profile E01 in Senegal and Guinea, but did not provide further details. This profile is identified in this present study in the south-east part of Niger (Diffa). The distribution of this profile could be explained by transboundary movement only if it was circulating in Mali or in the lake Chad region which share borders with Niger. The profile 'E' was also identified in Botswana but the study did not differentiate whether it was E01 or E02 (Kusiluka et al., 2001). This situation could be explained by the techniques used during this study (Amplified fragment length polymorphism: AFLP, and Pulsed-field gel electrophoresis: PFGE) which have weaker discriminating power than MLSA (Kusiluka et al., 2001).

The new *Mmm* profile named A15 by CIRAD differs from A00 by a new allele on loc-0001. The percentage nucleotide identity of the the new allele of the locus loc-0001 is 99% like the allele 4 of this locus. The difference is a substitution mutation at 282<sup>nd</sup> nucleotide, switching 'G' to 'A'. This mutation allows us to consider a seventh allele for the locus loc-0001. Similarities were also seen between the seven loci (i.e., loc-0103, loc-0287, loc-0431, loc-0489, loc-0710, loc-0523 and loc-0827) of the new profile A15 and the profile A00 (Table 3). These results can confirm Yaya's hypothesis, according

to which A00 could be a possible ancestral allelic profile, identified in strains isolated in Chad and the Gemu-Gofa region in Western Ethiopia (Yaya, 2008). This study also concluded the high variability at loc-0001 allele's level.

In summary, this study confirms the circulation of a new allelic profile A15 in Niger from 1990 to 2000. This profile was not early identified by previous studies (Yaya et al., 2008) likely due to the fact that there were no samples from Niger in his study. The majority of the profile identified belonged to profile A11 indicating the predominance of this profile in Niger. Finally, comparative sequence data have been generated for those interested in the circulation of *Mmm* in Niger and neighbouring countries and will assist veterinarian authorities in the implementation of CBPP disease control programmes in the country.

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#### CONFLICT OF INTEREST

The authors declared that there is no conflict of interests regarding the publication of this paper.

#### AUTHOR CONTRIBUTION

**Mahamadou Seyni Yansambou:** Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Writing-original draft; Writing-review & editing. **Maman Moutari SOULEY:** Formal analysis; Methodology; Software; Supervision; Writing-review & editing. **Alpha Amadou Diallo:** Formal analysis; Methodology; Software; Writing-review & editing. **Molefhi Abotseng:** Writing-review & editing. **Rianatou Alambédji-Bada:** Methodology; Supervision; Validation; Writing-review & editing.

#### COMPLIANCE WITH ETHICAL STANDARDS

In the respect of ethics, our study did not require the ethics committee approval in accordance with local legislation.

#### PEER REVIEW

The peer review history for this article is available at <https://publons.com/publon/10.1002/vms3.439>.

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#### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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